SYMPOSIUM ON PREPARATION OF SAMPLES FOR MICROBIOLOGICAL ASSAY[1]

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The precision and reliability of most of the commonly used microbiological assay techniques have been fairly well established. Many of the problems encountered in obtaining satisfactory analyses are due, not to the assay procedure *per se*, but to difficulties in preparation of the sample for assay. With the growing popularity of microbiological methods, there has been an increase in the complexity of the materials that are subjected to analysis, and consequent emphasis on the need for good extraction procedures. This symposium was organized to focus attention on some of the problems encountered, and to discuss a few of the specific methods which have been extensively studied.

Toepfer and Reynolds, whose paper was presented by their colleague, Dr. O'Barr, discussed the preparation of samples for vitamin assay, with particular reference to folic acid, pantothenic acid, vitamin B_6 and vitamin B_{12}. Working primarily with various foods, they found that, with the exception of vitamin B_{12}, enzymatic digestion of food samples generally was necessary to assure complete liberation of these vitamins in forms available to the assay organisms used.

Of several enzyme preparations tested for the release of folic acid from a variety of foods, chicken pancreas enzyme was found to be superior to papain, takadiastase or hog kidney enzyme. Using dried spinach as a substrate, they found naturally occurring enzymes in unheated suspensions which were about as effective as chicken pancreas enzyme in releasing folic acid. In samples heated to destroy the natural enzymes, 5 mg of chicken pancreas enzyme effected maximum release of folic acid from 1 gm of spinach. Samples could be autoclaved over a pH range of 5 to 13 without any loss of folic acid activity.

In the extraction of pantothenic acid from foods, a double enzyme system of alkaline intestinal phosphatase and pigeon liver enzyme was superior to mylase-P. Maximum release was observed when samples were incubated in the presence of both enzymes. A hog kidney enzyme preparation was found to be as effective as pigeon liver enzyme in the double enzyme system. Care must be exercised in the preparation and use of either of these enzymes to prevent loss of conjugase activity and consequent low pantothenic acid values. Evidence of the efficiency of the double enzyme system in releasing pantothenic acid from conjugates in several foods was provided by the results of parallel microbiological and bioassays.

In assaying selected food for vitamin B_6 activity with *Saccharomyces carlsbergensis*, autoclaving samples for 15 min with 0.055 N HCl, followed by overnight incubation with clarase or intestinal phosphatase was as effective as autoclaving for 5 hr in 0.055 N HCl in releasing this vitamin. These enzymes also were effective in releasing pyridoxal from pyridoxal phosphate.

Current evidence indicates that enzymatic digestion generally is not required in the preparation of samples for microbiological assay of vitamin B_{12}. There are conflicting reports, however, and studies currently in progress suggest that these may sometimes be attributable to liberation during enzymatic digestion of some foods, of materials that can replace vitamin B_{12} (deoxyribosides or deoxyribotides) for growth of *Lactobacillus leichmannii*.

The beneficial effect of certain antibiotics on the growth of farm animals has resulted in the marketing of antibiotic feed supplements and consequently the need for strict control of the antibiotic content of the feed. Tanguay pointed

[1] This symposium was held at the Fifty-seventh General Meeting of the Society of American Bacteriologists in Detroit, Michigan, on May 2, 1957. John Gavin, Chairman of the Committee on Analytical Microbiology, was the organizer. Participants were: Edward W. Toepfer and Howard Reynolds (represented by Thomas P. O'Barr), *Food Analysis Section, Human Nutrition Research Branch, United States Department of Agriculture, Washington, D. C.;* Alphee E. Tanguay, *Lederle Laboratories, Pearl River, New York;* and Lawrence J. Dennin, *Eli Lilly and Company, Indianapolis, Indiana.*

out that the complexity of modern feed formulations containing as many as 20 or 30 different ingredients has added greatly to the problems encountered in assaying feeds. Furthermore, it is expected that the rapidly increasing practice of adding medications directly to feeds will further complicate the assay problem. Tanguay discussed the effectiveness of various extraction techniques in removing chlortetracycline quantitatively from different types of feeds. Of the several procedures tested, his most consistent results were obtained following extraction of 20 g samples with 200 ml acid acetone for 3 min in a blendor or 1 hr on a rotary type shaker. Feeds contain extractable substances which interfere with the assay of chlortetracycline unless these substances also are added to the standard. In high potency feeds, 50 g per ton or above, the great dilution required usually makes the use of a blank feed standard unnecessary. In assaying low potency feeds, 10 g per ton or below, a blank feed standard was used to compensate for the feed extractables in the samples. Low recovery of chlortetracycline from a high potency commercial pig feed supplement was found to be due to its extremely high mineral content. Further work showed that most finished feeds contain extractable substances which protected chlortetracycline from inactivation by the high mineral content, so that the antibiotic was available to the animal in the complete feed. Satisfactory recovery of chlortetracycline in this instance could be achieved by (a) increasing the volume of acid acetone used to extract the sample so that these protective substances also were extracted; (b) chelation of the minerals with substances like 'Versene'; or (c) assaying against a blank feed standard or a standard with a mineral concentration equivalent to that of the sample.

Dennin discussed the factors that influence the selection of laboratory techniques and assay methods as applied to pharmaceutical preparations. Although the assayist working with pharmaceuticals has the advantage of relatively well defined samples, the modern trend toward combinations of active principals presents this worker with an increasing number of problems. The elimination of, or compensation for, interfering substances must be effectively dealt with if the assay values obtained are to be significant. In many instances, specific extraction procedures, inactivators or increment methods of analysis have to be developed to cope with the multiple vitamin or multiple antibiotic dosage form which is desired in the clinic. Each new dosage form developed presents a new challenge to the assayist.

Taken as a whole, the papers summarized here illustrate the many types of procedures required in preparing samples for assay and the versatility of thought with which the extraction procedure must be approached. One needs a thorough knowledge of the chemical nature of the substance to be assayed, the nature of the substrate from which it is to be removed, conditions that will effect a separation of the two and factors that will affect these conditions before considering what *could* be an effective procedure. The wealth of data presented by our speakers, which represented only a fraction of the actual work involved, was illustrative of the amount of effort required to find out what *does* constitute an effective extraction procedure.